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Our goal is the identification of genes that affect cellular response to an alkylating agent cyclophosphamide (CP): modulation of expression of these genes should protect cells from CP-induced cytotoxicity.

Identification of genes that increase/decrease cytotoxicity of CP will allow for better regimens of autologous bone marrow transplantation, since genetic modification of the patient's bone marrow cells before the first round of chemotherapy will reduce myelosuppression and will thus allow for better dose escalation.

We have identified four different genetic elements that dramatically increase cell survival after treatment with in vitro active CP analogue mafosfamide. One of genetic elements shares a high degree of homology with a nuclear protein kinase RING3; another is highly homologous to cytochrome oxidase subunit III; the other two elements do not have homology in the most recent releases of GeneBank. Protective effect of these elements is most pronounced when all four of them are present. Our current efforts are directed toward identification of full-length cDNA clones and studies of expression of corresponding genes.

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FOREWORD

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INTRODUCTION.

In this project we seek to identify genes that affect cellular response to alkylating agent cyclophosphamide (CP) and its derivatives; expression modulation of such genes will protect cells from CP toxicity.

Chemotherapeutic approach to advanced breast cancer treatment relies heavily on the use of alkylating agents, particularly CP. High-dose therapy in case of CP has to deal with several potentially dangerous side effects of this drug, myelosuppression being the most serious. Autologous bone marrow transplantation (ABMT) or peripheral blood stem cell transplantation (PBSCT) with or without hematopoietic growth factor support (HGFS) proved to be an effective adjunct therapy, which allows 10-20 fold increase in CP dose. This approach, however, has several serious drawbacks: (1) initial myelosuppression persists until engraftment is complete, leading to acute leukopenia immediately after drug treatment; this immunocompromised condition increases the risk of infectious complications; (2) although ABMT and PBSCT do not carry the risk of Graft-Versus-Host disease, the possibility of contamination with neoplastic cells increases with every consecutive round of transplantation, significantly reducing overall efficiency of the whole procedure; (3) ever-escalating cost of successive rounds of ABMT or PBSCT with HGFS increases financial burden on the patient and her family as well as society in general.

We have identified genetic suppressor elements (GSEs), which protect cells from toxic effects of in vitro analogue of CP mafosfamide (MA). These elements, when introduced in bone marrow in the course of the first round of ABMT/PBSCT, would protect bone marrow from CP, thus reducing or eliminating myelosuppressive effects of this drug and making additional rounds of ABMT/PBSCT unnecessary. ABMT/PBSCT with genetically modified, CP-resistant bone marrow (1) will allow rapid dose escalation without the risk of hematological complications; (2) will reduce the risk of re-introduction of neoplastic cells, imminent with repeated rounds of ABMT/PBSCT, and (3) significantly reduce the economic impact of the disease. Additionally, identification of genes involved in CP resistance will provide important insights into mechanisms of CP cytotoxicity and resistance and may also suggest new approaches for enhancing the antitumor effect of CP.

Advanced breast cancer is currently associated with extremely poor prognosis when both hormone treatment and chemotherapy are considered palliative rather than curative treatments (reviewed in 1). Hormone therapy is the first line of defense, and it can be used if cancer cells retain functional estrogen and progesterone receptors and visceral tumor nodes are absent (2). However, a rapid decrease in the response rate to hormonal therapy ultimately necessitating chemotherapy, usually with a combination of several drugs. The standard chemotherapeutic regimens for breast cancer are based on doxorubicin (often used as a single agent) and CMF, which consists of an alkylating agent cyclophosphamide (CP), 5-fluorouracil, which inhibits thymidilate synthase, and dihydrofolate reductase inhibitor methotrexate (3). This well-established regimen is sometimes augmented by mitoxantrone (4), leucovorin (5), thiotepa (6), pentoxifylline (7), etoposide (8), etc., but these compounds are used much less frequently, and their ability to replace the agents of the CMF regimen is still under investigation (see 9 for a review).

Cyclophosphamide - an alkylating agent from oxazaphosphorine family - requires activation by hepatic microsomal enzymes; its active form - 4-hydroxycyclophosphamide (4-HC) - is released in blood stream and transported throughout the body. Entering the cell

4-HC is converted into aldophosphamide which decomposes into phosphoramide mustard (PM) and acrolein. Alkylation of DNA by PM - guanine at position O^6 or adenine at position 3 - induces either point mutations (in case of O^6-alkylguanine, 10) or DNA breaks through apurinic/apyrimidinic sites (in case of 3-alkyladenine, 11). PM can also induce inter- and intrastrand DNA crosslinks as well as DNA-protein crosslinks (12) while acrolein has been implicated in single-strand breaks in DNA (13).

Biochemical modifications induced by 4-HC can either inhibit cellular proliferation until the damage is repaired or can promote initiation of an active cell death program; in the latter case the affected cell will be destroyed. The outcome of treatment will depend on three major factors: intracellular concentration of the drug, availability of the target and the ability to repair cytotoxic damage. Drug concentration in its turn is affected by the rates of uptake and efflux, drug's chemical stability and its detoxification by cellular enzymes.

One of the obvious means to increase effective intracellular drug concentration is the escalation of the administered dose, and this approach remains one of the most frequently used tactics in cancer chemotherapy. Unfortunately, chemotherapeutic agents are essentially toxins which affect normal tissues as well as malignant neoplasias, and toxic side effects can become unacceptable during and after drug treatment. Furthermore, even the maximum tolerated dose (MTD) of any drug gives just temporary improvement, since neoplastic cells usually become resistant to a particular class of cytotoxic compounds, so that subsequent treatments - however toxic to the patient - would no longer produce any deleterious effect on the tumor itself (14). Resistance to chemotherapy develops gradually and depends on alterations in genome functions (e.g. induction of MDR1 expression which leads to increased efflux of some drugs thus reducing their intracellular concentration, 15-17); in case of CP known mechanisms of resistance involve elevated levels of glutathione (18), increased activity of glutathione-S-transferase (19), aldehyde dehydrogenase (ALDH, 20) and gamma-glutamyl transpeptidase (21). Genetic regulation of resistance implies that genes involved can be studied and used either to block such resistance in tumor cells and thus make them susceptible to chemotherapy, or to protect normal cells against chemotherapeutic side effects. Obviously, increasing the level of resistance in normal cells would allow rapid dose escalation and increase the overall efficiency of chemotherapeutic treatment.

In advanced breast cancer high-dose chemotherapy is an effective strategy to significantly reduce tumor burden in cancer patients. Hyrnuik et al. (22, 23) observed direct correlation between dose intensity of CP and remission rate in advanced breast cancer; clearly, the higher the dose of initial chemotherapeutic challenge the better are chances of remission. CP dose increase is limited by side effects on bone marrow, uro-, cardio- and pulmonary toxicity as well as terato- and oncogenic effects (24); myelosuppression is the major problem since hematopoietic cells are extremely sensitive to the toxic effects of CP and other chemotherapeutic drugs (25). Severe leukopenia and granulocytopenia as a result of CP treatment increases patient's susceptibility to pathogens and opportunistic bacteria: infectious complications, including septicaemia, are common in patients after high dose of CP (26). Reconstruction of hematopoietic stem cell population after high-dose chemotherapy is realized by autologous bone marrow transplantation (ABMT) or peripheral blood stem cell transplantation (PBSCT), coupled with administration of hematopoietic growth factors. This approach permits significant (10-20 fold, 27) dose escalation with relatively low morbidity and considerable increase in disease-free survival (28). A serious drawback of high-dose chemotherapy in combination with ABMT/PBSCT is the cost of the

procedure ($48,000 to $384,000, 29); considering that such treatment has to be administered repeatedly the overall economic impact in some cases becomes prohibitive (29, 30).

Increased resistance of hematopoietic stem cells to CP treatment would lead to reduction in both the morbidity rate and cost of the treatment. It would allow rapid dose escalation without adverse effects on hematopoietic system, reduce or even eliminate the risk of infectious complications after treatment, and eliminate the need for repeated ABMT/PBSCT. In this project we have identified genetic elements which provide cell protection from cytocidal effects of CP and its derivatives. Such elements can be used for gene therapy as well as for determination of mechanism(s) of resistance against CP and its analogues. Currently, ALDH has been suggested for gene therapy application as a potential chemoprotectant against CP: transfection of ALDH was reported to provide several-fold increase in resistance to CP anlogues (31). Whether ALDH-conferred modest resistance to CP will be applicable and/or sufficient for hematopoetic cell chemoprotection remains to be established.

To identify genetic elements that can protect cells against CP and its derivatives, we used an approach developed in the laboratory of Dr. Igor Roninson. This method is based on the regulation of gene function by genetic suppressor elements (GSEs), short gene fragments that encode biologically active antisense RNAs or short peptides (32). GSEs are isolated by selection for a desired phenotype using an expression library of randomly fragmented cDNA, which may correspond to a single cDNA clone (33) or a population of all cDNA sequences (34, 35). This approach has been successfully used to identify genes that mediate the cytotoxic effect of different anticancer drugs, since GSEs arising from such genes act as selectable markers of drug resistance. Thus, GSEs selected from the cloned cDNA of topoisomerase II produced resistance to different drugs that interact with this enzyme (33). Selection for resistance to etoposide from a library carrying random fragments of a normalized (uniform-abundance, 36, 37) cDNA from a mammalian cell line resulted in the isolation of resistance-inducing GSEs from the heavy chain of kinesin and from two previously unknown genes (34). Importantly, some of the GSEs selected with individual drugs produced resistance to other, unrelated agents. Thus, a kinesin-derived GSE, originally selected with etoposide, conferred resistance to several DNA-damaging drugs and also promoted immortalization of senescent mouse embryo fibroblasts (34).

In this study, we have used a normalized human cDNA fragment library to isolate GSEs conferring resistance to a CP analogue, mafosfamide (MA). We identified a set of genetic elements, which can be used for gene therapy and also characterized new genes involved in CP response.

BODY OF THE REPORT

Experimental methods, assumptions and procedures.

The experimental approach used in this study consists of two parts: (1) identification of GSEs, which confer resistance to CP in human fibrosarcoma cell line HT1080 and (2) evaluation of their effects in cell line K562 (chronic myelogenous leukemia). Primary selection and initial testing have been done with MA - an analog of CP, which does not require microsomal activation and therefore can be easily used for in vitro studies (38). Individual GSE, capable of protecting cells from MA, were to be tested against hepatocyte-activated CP, which was thought to be a better approximation of in vivo situation. The initial selection was carried out in HT1080 fibrosarcoma cells, and the selected GSEs were to be tested in K562 leukemia cells.

Aim 1. Introduction of normalized library of random cDNA fragments cloned in retroviral expression vector LNCX into HT1080 cell line and selection for GSEs conferring resistance to CP derivative mafosfamide.

Normalized library of short (250-400 bp) fragments of cDNA cloned in retroviral expression vector LNCX (39), as well as HT1080/Eco cell line was provided by Dr. Igor Roninson. LNCX contains a dominant selectable marker (neomycin phosphotransferase) which allows efficient selection of infected cells in G418-containing medium (39). This library was transfected by standard calcium-phosphate co-precipitation procedure into ecotropic packaging cell line BOSC 23 (40) to generate infectious viral particles, which were used to infect HT1080/Eco cells. Transduction of LNCX without an insert was used as a control. Efficiency of transduction was determined by (a) infection and G418 selection of NIH 3T3 fibroblasts to evaluate viral titer; and by (b) selection of a defined number of infected HT1080/Eco in G418-containing media. Number of G418-resistant clones obtained versus plating efficiency served as a measure of transduction efficacy. In our hands 25-45% of the HT1080/ETR cells can be infected by this method; for library selection $3x10^7$ cells have been infected to ensure complete representation of the library (its complexity is estimated to be approx. 10^7).

Selection of HT1080/Eco cells was performed by long-term (24 hr) exposure to MA. This treatment was chosen to simulate continuous drug application in the chemotherapy regimen. The application of MA, which resulted in survival of 1-5 out of 10^5 LNCX-transduced cells, was chosen for selection. Resistant colonies after the first round of selection were expanded, and their genomic DNA was used for PCR-mediated recovery of inserts. These inserts were directionally cloned into LNCX vector, and this second-order library was used for an additional round of selection (see Fig. 1). Non-identical individual clones were tested for their ability to confer resistance to MA through BOSC 23 transfection, infection of the target cells and selection with MA.

The initial hypothesis suggested that (1) individual GSEs would provide protection against MA selection and (2) multiple rounds on infection-selection-insert recovery would allow us to reduce the complexity of the library to the point when every insert would

provide resistance. Both of these assumptions turned out to be incorrect (see results and discussion), and significantly more time was spent at this stage of the project. Although this setback prevented us from carrying out Aims 2 – 4, we identified GSEs, which could protect HT1080 cells against MA (see results and discussion).

Aim 2. Introduction of GSEs conferring resistance to mafosfamide in HT1080 cells into K562 cell line and evaluation of their activity.

Individual GSEs (in LNCX vector), which could protect HT1080/ETR cells from MA were to be introduced into K562 cells by transduction. Cells infected with vector without an insert were to serve as a control. Conditions of MA exposure, which result in complete or almost complete death of the control population, were to be determined. Cell survival in this case were to be monitored by colony-forming assay after plating 10^4 drug-treated cells in the medium containing 0.3% Seaplaque agarose (13); as a control the same population were to be grown in the presence of G418. Survival curves were to be determined for each infected population with the LNCX-infected population as a control.

Aim 3. Protection against activated CP by GSEs that induce MA resistance.

Individual GSEs, which could protect HT1080/ETR and K562 cells from MA, were to be introduced into HT1080/ETR cells as described in Aim 1 and into K562 cells as described in Aim 2. In vitro activation of CP were to be done as in (13), except that human hepatoma cell line Hep G2 were to be used instead of primary hepatocytes; Hep G2 has been shown to be metabolically competent to activate CP (42). Briefly, different concentrations of CP were to be added to 10^6 Hep G2 cells in 60 mm plate for 1 hr; after incubation, the culture medium were to be removed and filtered to eliminate stray cells. Filtered media containing activated CP were to be added to log-phase HT1080 or K562 cells for 24 hr. Colony assay were to be done as described above (see Aims 1 and 2). Survival curves were to be determined for each infected population with LNCX infected population as a control.

Aim 4. Identification of genes that give rise to GSEs protecting K562 cells from mafosfamide and hepatocyte-activated CP.

Individual GSEs, which confer resistance to MA and hepatocyte-activated CP in both HT1080 and K562, were to be used as probes to screen a full length human cDNA library (commercially available, e.g. from Stratagene). Positive clones were to be sequenced by conventional methods and compared with sequences from Genebank.

Results and discussion.

The key element in this project was identification of individual GSEs, which could confer resistance to MA when introduced into HT1080/Eco. Accordingly, considerable effort has been devoted to identification of these GSEs and their functional testing in the cell. Our initial results indicated that transduction of GSE library can induce significant resistance to MA (Fig. 2). Several enriched elements were selected from the secondary

library for subsequent individual testing; individually, however, these GSEs failed to induce any protection in freshly transduced cells, while additional tests with enriched library confirmed the presence of protective elements. These results forced us to change the initial hypothesis in favor of possible cooperative action of transduced GSEs. To test this hypothesis, we isolated individual cellular clones after multiple library transduction and selection with MA. Analysis of GSEs recovered from resistant clones indicated that all of them contain several (up to six) different inserts; four of them were represented in the majority of clonal sub-libraries (Fig. 3). Re-introduction of these four GSEs resulted in significant resistance of transduced cells to MA (Fig. 4).

Additional experiments, performed to test the modified hypothesis, have significantly delayed our project. Nevertheless, we have identified protective GSEs and therefore the primary goal of the project can be considered as completed. At this time we have finished Technical Objective 1, Task 5 of original SoW (Evaluation of the protection against MA by selected GSEs); Technical Objective 4, Task 8 (Identification of genes that give rise to protective GSEs) is currently underway.

Technical Objectives 2 and 3 (Evaluation of protective effect against MA in K562 and Evaluation of protective effect against hepatocyte-activated cyclophosphamide in K562 and HT1080) have not been performed due to the conceptual problem outlined above.

CONCLUSIONS.

GSE approach allows identification of genes involved in cellular response to various drugs, and the main goal of the project – isolation of such GSEs – has been accomplished. Initial hypothesis, however, postulated that individual GSEs would be capable of inducing resistance to MA at a sufficient for selection level. Accordingly, we carried out several steps of library transduction – selection – recovery and analyzed individual GSEs, selectively enriched in this process. No individual elements tested could protect cells against MA.

Modification of the initial hypothesis, which allowed for selection of a combination of GSEs, led to identification of four elements, which can significantly reduce cellular sensitivity to MA. Corresponding cDNAs will be isolated shortly.

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APPENDICES.

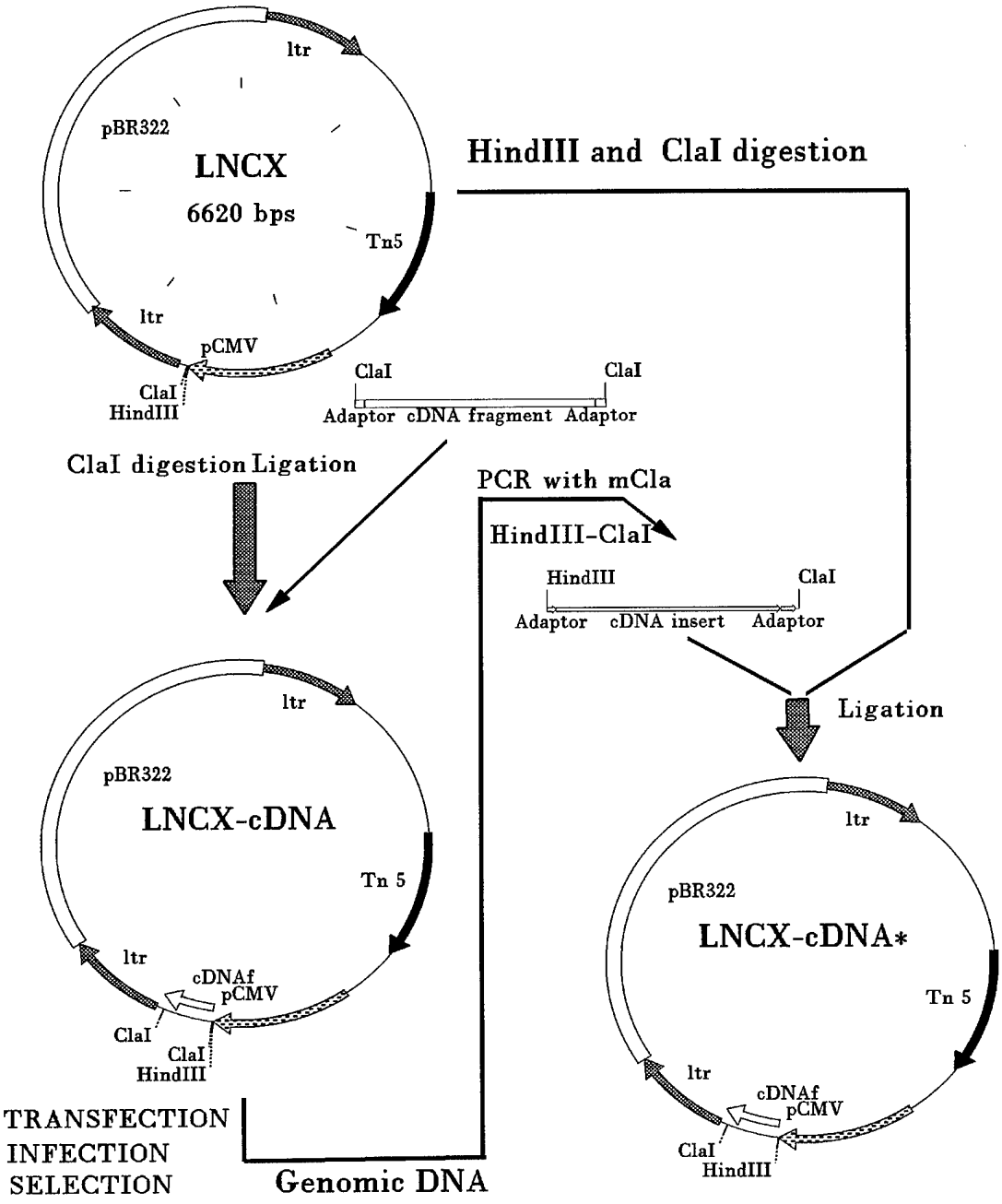


Fig. 1. Cloning steps in GSE selection procedure. PCR primers used for genomic PCR allow directional cloning of the GSEs.

Library LNCX

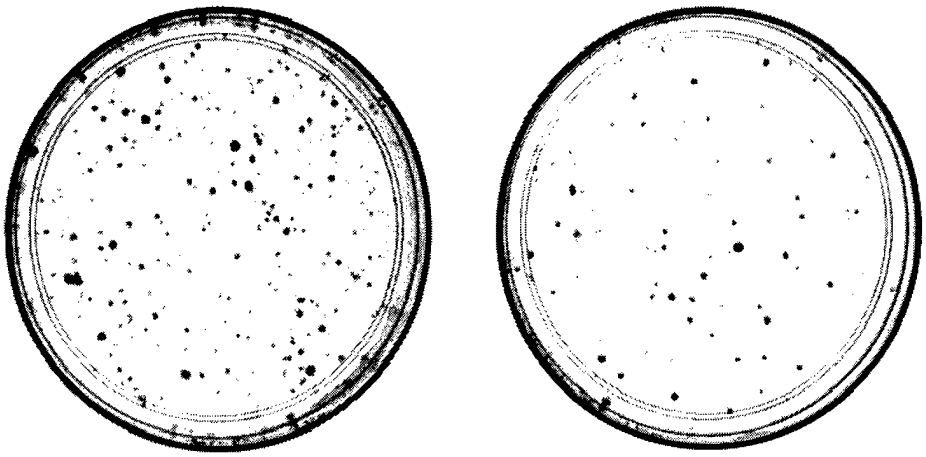
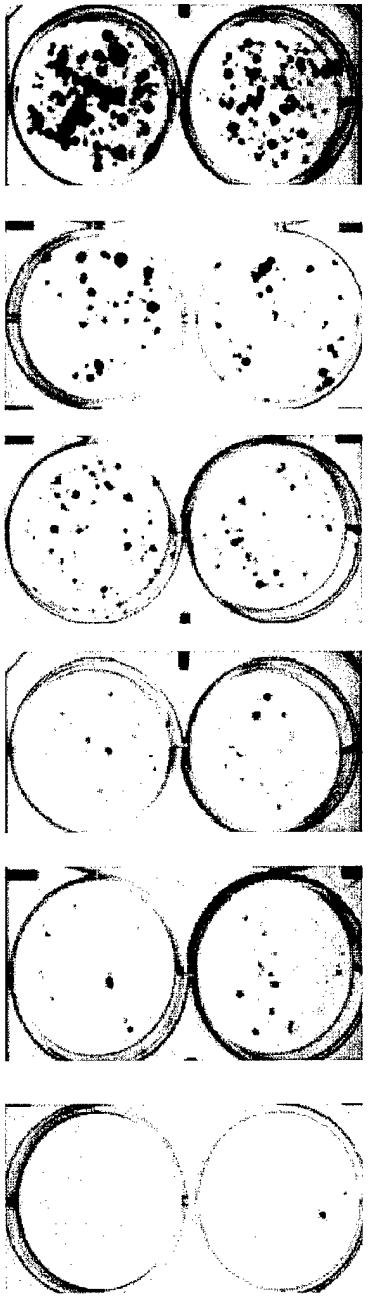


Fig. 2. Selection with MA on HT1080/Eco cells transduced with GSE library or with vector alone.



A1	A8	B5	C5
+	+	+	+
+	+	+	
+	+	+	
+	+	+	
	+	+	+
N	O	N	E

Fig. 3. A combination of GSEs is present in resistant clones.

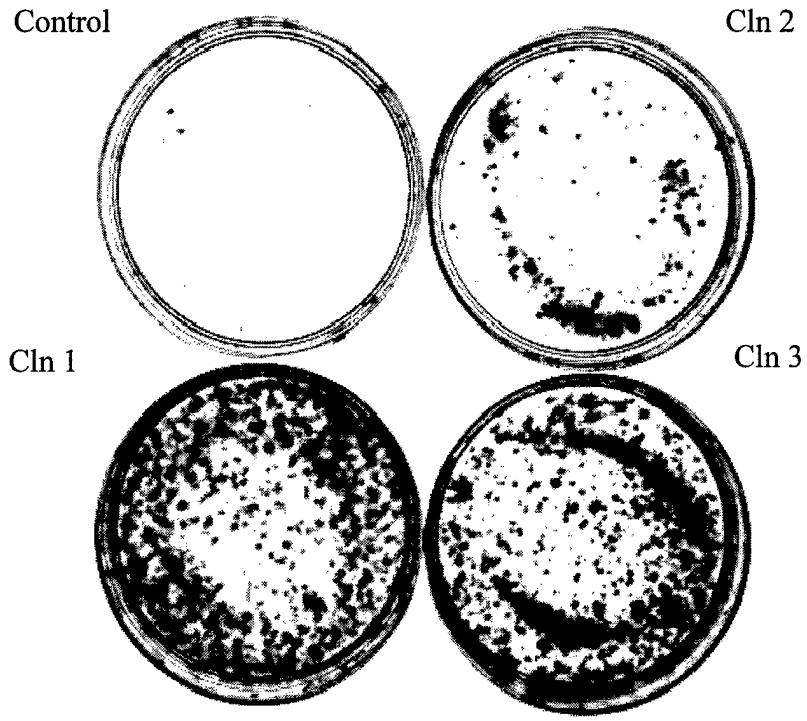


Fig. 4. Re-introduction of GSE combination confers MA resistance to transduced cells.